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14. ABSTRACT

Mutations in the human TSC2 gene cause tuberous sclerosis complex (TSC), a developmental disorder characterized by tumor susceptibility and neurological manifestations. To better understand the disease we generated an animal model in which the mouse Tsc2 gene is disrupted exclusively in excitatory neurons of the forebrain. We are investigating how heterozygous and homozygous Tsc2 mutations affect the development of mutant excitatory neurons as well as other surrounding brain cells, in vivo and in vitro. During this second year of support we focused on Major Task 1: In vivo characterization of heterozygous and homozygous NEX-Tsc2 mice. Specifically, we performed experiments described under Subtask 1: Animal breeding for in vivo studies, Subtask 3: Analysis of neuronal development and synaptogenesis, Subtask 4: Analysis of non-cell autonomous differentiation and signaling defects, and Subtask 6: Epilepsy and seizure susceptibility. We found that heterozygous mutations of Tsc2 in the excitatory neurons of the forebrain have no detectable effects on neuronal differentiation but increase seizure susceptibility, whereas homozygous loss disrupts the maturation not only of excitatory neurons (cell-autonomous) but also disrupts the development of inhibitory synapses (non cell-autonomous effect) and cause recurrent seizures.

15. SUBJECT TERMS
Tuberous Sclerosis Complex, animal model, neuronal development, synapse, TSC2

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1. Introduction

Mutations in the *TSC2* gene cause tuberous sclerosis complex (TSC), a developmental disorder characterized by tumor susceptibility in multiple organs and frequent neurological manifestations, such as seizures, intellectual disability, and autism. There is currently no cure for TSC, although significant progress has been made in recent years in managing some of the clinical traits, particularly tumor growth and epilepsy. However, much remains to be done to relieve the burden of epilepsy and cognitive problems, such as intellectual disability and autism. In line with the mission of TSCRP, the goal of this research is to improve our understanding of the pathogenesis of TSC, focusing on the cellular and molecular mechanisms that lead to the development of neurological symptoms. We believe that this basic knowledge can be translated into better treatments, and can ultimately improve the lives of TSC patients.

We hypothesized that heterozygous mutations in the *TSC2* gene disrupt the normal development and function of excitatory neurons without affecting their size. Homozygous loss-of-function mutations, on the other hand, not only profoundly alter their size and intrinsic development, but also disrupt cell-cell communication with other cell types. These non cell-autonomous mechanisms exacerbate defects in synaptic function and cognition, and possibly contribute to the formation of cortical tubers and tumors in the TSC brain.

2. Keywords

Tuberous Sclerosis Complex, animal model, neuronal development, synapse, TSC2

3. Accomplishments

- **Major goals of the project**

The overall goal of this study is to define the cellular abnormalities of excitatory neurons that are deficient in TSC2 activity, and to understand how they impact the development of other neuronal and glial subtypes in the cerebral cortex.

For our study we utilize NEX-*Tsc2,* a conditional *Tsc2* knock out mouse line in which gene deletion occurs specifically in embryonic forebrain excitatory neurons, the most abundant cell type in the developing cerebral cortex. We proposed to characterize the neurodevelopmental defects of heterozygous and homozygous NEX-*Tsc2* mice, including cell autonomous abnormalities in the development of excitatory neurons, as well as non-cell autonomous effects on inhibitory neurons and non-neuronal cell types. In collaboration with Dr. Anne Anderson, we also planned to use video-EEG recordings to determine whether heterozygous and homozygous NEX-*Tsc2* mice exhibit seizures or increased susceptibility to chemoconvulsants. Our proposed work includes in vivo studies (Specific Aim1), some of which were performed during this period, and in vitro culture studies (Specific Aim 2), which will be performed as planned in the upcoming year of support.

As detailed in the SOW, during this second year of support we focused on Major Task 1: *In vivo* characterization of heterozygous and homozygous NEX-*Tsc2* mice, corresponding to Specific Aim 1 of the research proposal.

- **Accomplishments under these goals**

Subtask 1: Animal breeding for *in vivo* studies.

We generated experimental NEX-Tsc2 mice by breeding $Cre^{+/+}$;$Tsc2^{flox/wt}$ mice with heterozygous mice $Cre^{-/-}$;$Tsc2^{flox/wt}$. This breeding strategy generated 100% $Cre^{+/-}$ mice that were either wild type (WT = $Tsc2^{wt/wt}$), heterozygous (HET = $Tsc2^{fl/wt}$) or homozygous (KO = $Tsc2^{fl/fl}$) for *Tsc2*. Pups were born at the expected Mendelian ratio (25% WT, 50% HET, and 25% KO). As expected HET mice appeared indistinguishable from WT controls, whereas KO mutant mice appeared runt and some died prematurely at approximately 12-15 days after birth (P12-15). Despite some mortality, we were able to obtain multiple sets of mice of each genotype (WT, HET and KO) for our molecular analysis. To maintain the colony and regenerate breeder mice of the appropriate genotype we also interbred separately $Cre^{+/+}$;$Tsc2^{flox/wt}$ mice as well as $Cre^{-/-}$;$Tsc2^{flox/wt}$ and selected $Tsc2^{flox/wt}$ from each progeny. These breeders were regenerated twice per year to ensure fertility.

Subtask 3: Analysis of neuronal development and synaptogenesis.

Experiments conducted during the first year of support indicated that pan-neuronal markers such as MAP2, TuJ1, Tau and NFH we not significantly altered in the whole cortical lysates prepared from heterozygous or homozygous NEX-Tsc2 mice. Also, synaptic markers such as Synaptophysin (Syn) and PSD95 were not altered, but there seemed to be a decrease in the inhibitory synapse-specific marker Gephyrin specifically in homozygous mutants. To confirm and expand on this observation we conducted RT-PCR analysis of neuronal markers, re-run protein samples and examined additional synaptic markers in both whole lysates as well as crude synaptosome preparations by Western blot analysis. RT-PCR analysis was conducted after extracting total RNA samples from the cerebral cortex or the hippocampus of P16 NEX-Tsc2 mice using gene specific primers. Specific proteins were detected using the quantitative LI-COR Odyssey Fc imaging system. Blots will be stained with REVERT™ Total Protein Stain (Li-COR) to control for protein loading, followed by primary antibodies and secondary antibodies conjugated to near-infrared fluorophores (Li-COR). Statistical analysis was performed by ordinary one-way ANOVA with Dunnett's post-hoc multiple comparisons using the GraphPad Prism7 software.

RT-PCR analysis of neuronal markers in the forebrain of NEX-Tsc2 mice.

The data plotted below show that there is a significant decrease in *Tsc2* mRNA levels in heterozygous and homozygous mutant both, in the cerebral cortex (Fig. 1) and in the hippocampus (Fig. 2). However, there was no significant difference in the expression levels of the neuronal markers βIII Tubulin (*TuJ1)* (p>0.05). The data are consistent with our previous Western blot data.

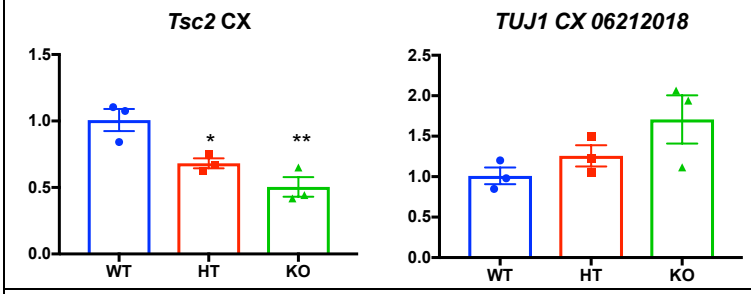

Figure 1. RT-PCR analysis of Tsc2 and TuJ1 expression in the cerebral cortex. One-way ANOVA with Dunnett's multiple comparisons, n=3 samples per genotype, *p<0.05, **p< 0.01.

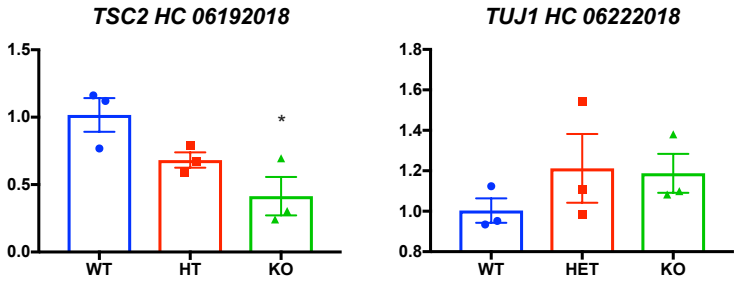

Figure 2. RT-PCR analysis of Tsc2 and TuJ1 expression in the hippocampus. One-way ANOVA with Dunnett's multiple comparisons, *n*=3 samples per genotype, *p<0.05.

Western blot analysis of synaptic markers in whole forebrain lysate of NEX-Tsc2 mice.

The data below show that there is no significant difference between genotypes regarding the expression levels of general presynaptic marker Synaptophysin (Syn), inhibitory-specific presynaptic marker VGAT and Synaptotagmin2 (Syt2), excitatory-specific postsynaptic marker PSD95 and inhibitory-specific postsynaptic marker Gephyrin. Nevertheless, the expression levels of most of these markers appeared consistently decreased in homozygous mice. However, there was a significant decrease in the levels of the excitatory-specific presynaptic marker VGlut2. These results suggest that Tsc2 deficiency in excitatory neurons results in a strong deficit in the formation of excitatory synapses, but it may also affect other synapses.

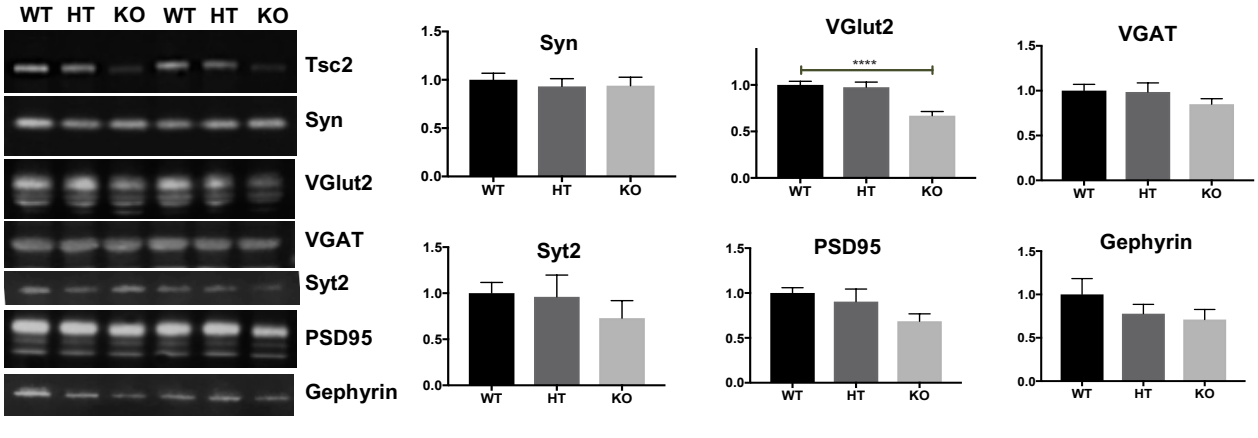

Figure 3. Western blot analysis of synaptic markers in whole forebrain homogenates. One-way ANOVA with Dunnett's multiple comparisons, *n*=5 samples per genotype, ****p<0.0001.

Western blot analysis of synaptic markers in synaptosomes of NEX-Tsc2 mice.

To examine synaptic markers in more detail we prepared crude synaptosome fractions of the forebrain of P16 NEX-Tsc2 mice and conducted Western blot analysis of the same markers. The data confirm that there is no significant difference between genotypes in

the expression levels of general presynaptic marker Synaptophysin (Syn) or inhibitory-specific presynaptic marker VGAT, but there is a significant decrease in the excitatory-specific presynaptic marker VGlut2 and the inhibitory-specific marker Synaptotagmin2 (Syt2) in homozygous mice. Also, the data confirm that excitatory-specific postsynaptic marker PSD95 is not altered, but indicate that inhibitory-specific postsynaptic marker Gephyrin is decreased, although the difference was significant only when homozygous values were compared to heterozygous values. Overall the results so far suggest that Tsc2 deficiency in excitatory neurons results in a strong deficit in the formation of excitatory synapses, and possibly also affect inhibitory synapses.

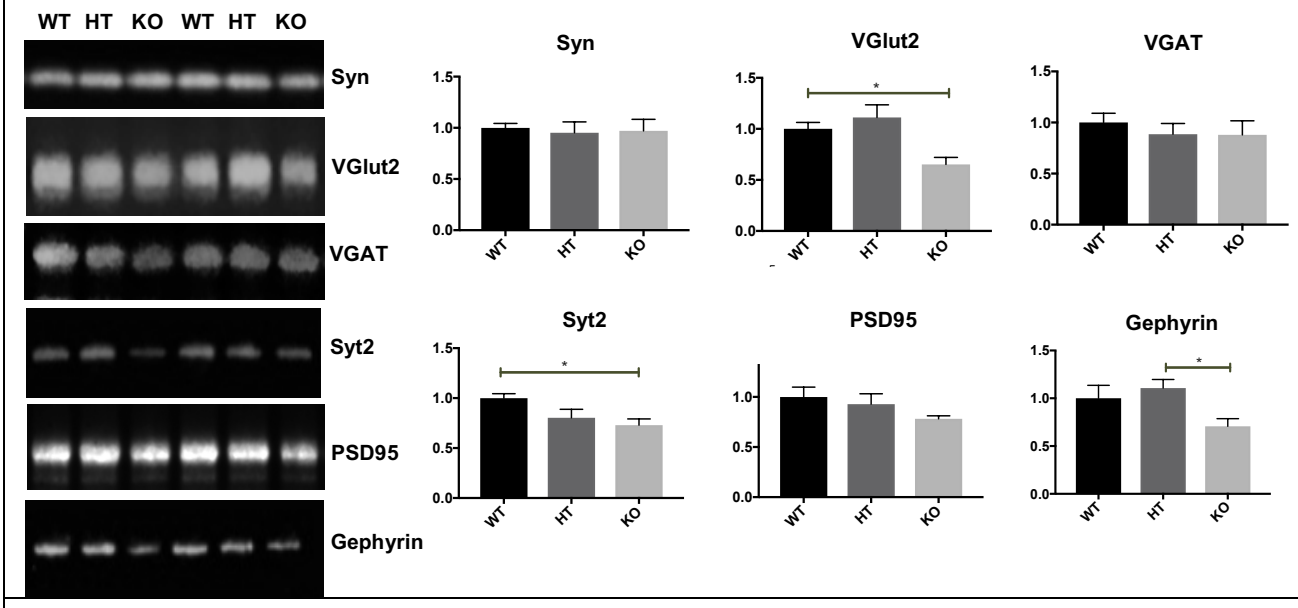

Figure 4. Western blot analysis of synaptic markers in synaptosome fractions. One-way ANOVA with Dunnett's multiple comparisons, *n*=5 samples per genotype, except for Syn (*n*=3-4 samples per genotype), *p<0.05.

Subtask 4: Analysis of non-cell autonomous differentiation and signaling defects

The data above indicate that the Tsc2 mutation in homozygosity not only disrupts the development of excitatory neurons (cell-autonomous) but it may also interfere with the development of interneurons forming inhibitory synapses on mutant cells (non cell-autonomous). To explore this possibility, we conducted RT-PCR and Western blot analysis of GAD67 (an inhibitory neuron marker) mRNA and protein expression in the forebrain of P16 mice.

RT-PCR analysis of *GAD1* (GAD67) in the forebrain of NEX-Tsc2 mice.

The data below show that there is no significant decrease in *GAD1 (GAD67)* mRNA levels in heterozygous and homozygous mutant both, in the cerebral cortex or hippocampus (Fig. 5). However, there was a modest decrease in the homozygous hippocampus.

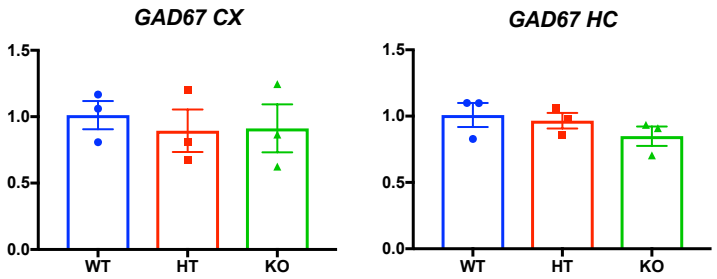

Figure 5. RT-PCR analysis of the *GAD1* gene (encoding GAD67) expression in the cerebral cortex (CX) and hippocampus (HC). One-way ANOVA with Dunnett's multiple comparisons, *n*=3 samples per genotype.

Western blot analysis of GAD67 expression in whole forebrain lysate of NEX-Tsc2 mice.

To examine the levels of GAD67 protein we conducted Western blot analysis of the same whole forebrain homogenate samples used for the above analysis of synaptic markers and some previous samples. The data show that there is a modest decrease in GAD67 expression in homozygous samples. However, the significance of these results depends on the statistical tool utilized. One-way ANOVA comparing all 3 genotypes indicates a marginal but not significant difference (p= 0.0574), whereas an unpaired t test comparing only WT to KO samples indicates a significant difference (p=0.0197).

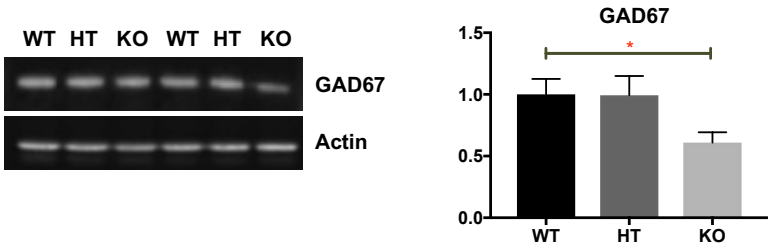

Figure 6. Western blot analysis of GAD67 expression in whole forebrain homogenates. One-way ANOVA with Dunnett's multiple comparisons (p>0.05), unpaired t test WT versus KO *p<0.05, *n*=8-9 samples per genotype.

Immunofluorescence analysis of GAD67 expression in the forebrain of NEX-Tsc2 mice.

To further investigate the expression of GAD67 in the forebrain we perfused 3 sets of P16 NEX-Tsc2 mice, prepared brain sections with a cryostat, and conducted immunofluorescence. Epifluorescence images reveal the presence of sparse GAD67+ cells in the cortex and hippocampus of WT and KO mice. Confocal images further revealed the cell bodies of these GAD67+ inhibitory neurons as well as their presynaptic terminals decorating the cell bodies of GAD67- excitatory neurons in both brain regions. At first glance GAD67 expression appears normal in KO mice. Further quantitative analysis will be required to conclusively determine whether there are any significant differences between genotypes.

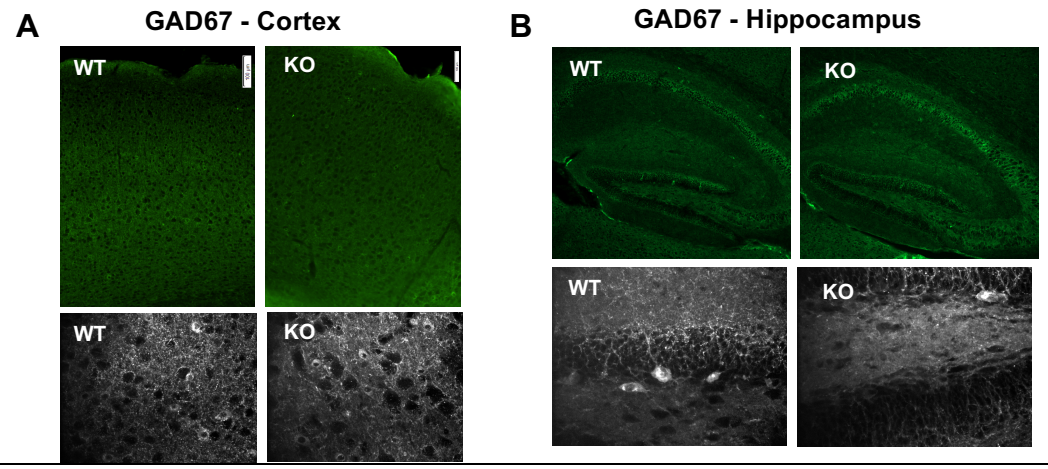

Figure 7. Immunofluorescence analysis of GAD67 expression in the cerebral cortex (A) or hippocampus (B) of NEX-Tsc2 mice. Top panels (green) are epifluorescence images at 10x magnification; bottom panels are confocal images at the 20X magnification.

Subtask 6: Epilepsy and Seizure Susceptibility

Seizures and EEG abnormalities.

Recurrent spontaneous seizures were evident in KO mice beginning at P11 and observed until death (Figure 8A). No epileptiform activity was detected in WT or HT mice. Epileptiform activity in KO mice appeared initially as isolated interictal spike activity that evolved into frequent polyspike discharges. Whereas exploratory time in KO mice was normal on the first day of recording, subsequent days consisted of longer periods of immobility interrupted by brief periods of exploration. Immobility coincided with epochs of epileptiform activity. Quantitative EEG analysis revealed that total EEG power (0.5-50 Hz) was decreased compared to WT in KO from P11 to P14 and in HT mice at P14 (Figure 8A, 8B, and 8C). KO mice showed significantly reduced power in delta frequencies at P11 compared to WT and HT mice (1-3 Hz; Figure 8C), while HT are comparable to WT at this age. At P14, spectral power was significantly reduced in delta and theta frequencies in both KO and HT relative to WT (KO: 1-6 Hz; HT: 1-4 Hz; Figure 8D).

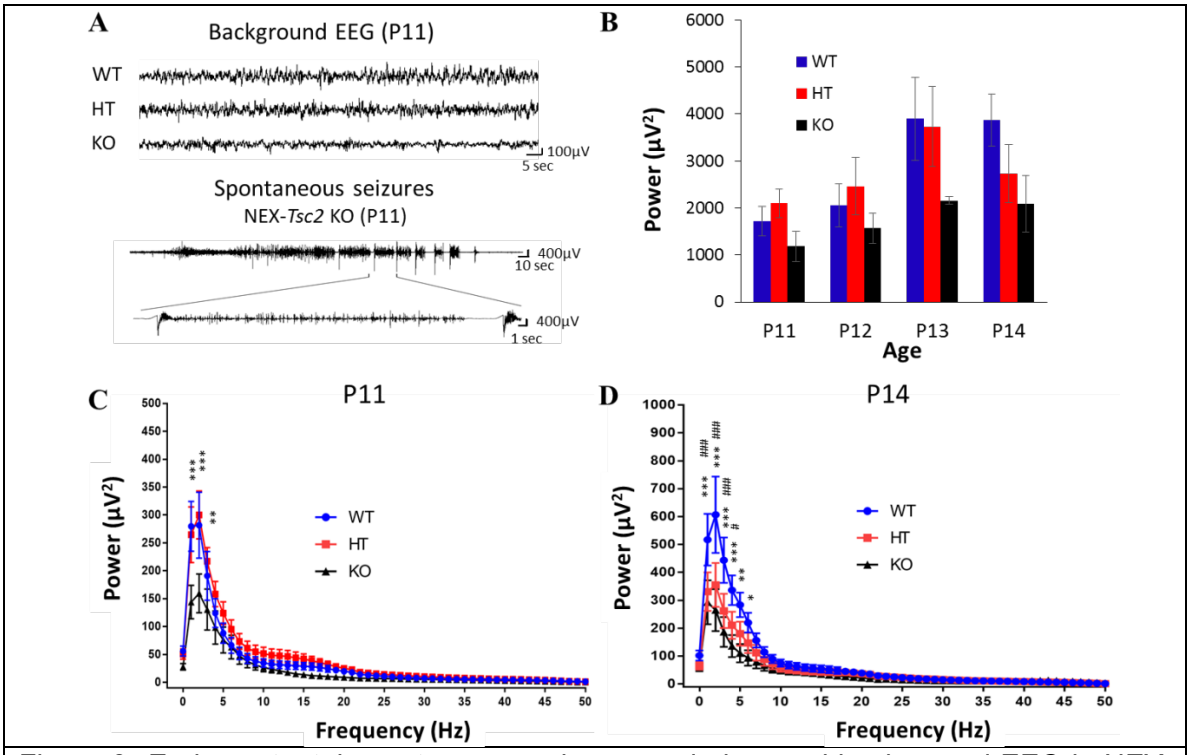

Figure 8. *Early postnatal spontaneous seizures and abnormal background EEG in NEX-Tsc2 mice.* A) Representative cortical EEG from WT, HT, and KO mice at P11 as well as EEG activity from a KO during a spontaneous tonic-clonic seizure. B) Total cortical power (0.5-50 Hz) from WT, HT, and KO mice at P11-P14. Spectral power analysis at C) P11 and D) P14 showed a decrease in delta frequencies in KO mice at P11. At P14, reduced power compared to WT is found in an increased range of frequencies in KO mice, while HT mice showed a reduction in delta frequencies compared to WT mice. Two-way ANOVA; WT vs KO: *p<0.05, **p<0.01; ***p<0.001; WT vs HT: #p<0.05, ###p<0.001; P11: n=6-10/group; P14: n=4-10/group; Data represent mean ± SEM.

Seizure susceptibility in NEX-Tsc2 mutant mice.

To assess seizure threshold, separate cohorts underwent seizure induction with the chemoconvulsant pentylenetetrazol (PTZ; 50mg/kg intraperitoneal) at P12 or P15 (Figure 9). KO mice are more vulnerable than WT and HT mice. Almost all KO mice at both age groups enter status epilepticus (Figure 9C&D), fail to recover and continue to exhibit electrographic seizure bursts and severe tonic clonic seizures without regaining posture even at 1 hour post PTZ (Figure 9A and 9B). Latency to the first seizure varied widely between 1 and 10 minutes with no difference among genotypes.

Data from our pilot experiments at P49 showed HT mice were more susceptible to PTZ than WT mice. Although all mice from both groups developed tonic-clonic seizures after PTZ, 60% of HT mice died whereas all WT mice recovered within 1 hr (n =5 WT, 7 HT). EEG analysis at this time revealed that surviving HT mice displayed an increased frequency of rhythmic spiking compared to WT mice.

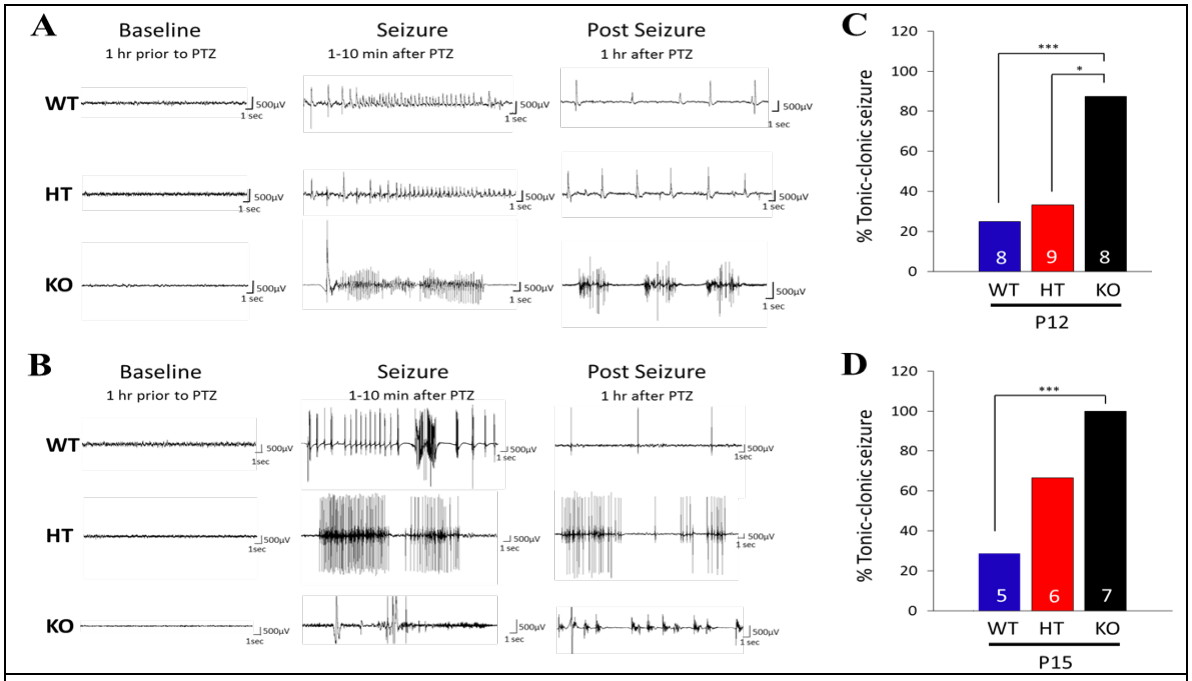

Figure 9. *Increased PTZ seizure susceptibility in juvenile NEX-Tsc2 mutant mice.* After a single injection with PTZ, mice were observed for 1 hour with seizure activity scored using EEG activity (A,B) and behavior (C, D) in mice age P12 (A, C) or P15 (B, D). EEG activity was recorded before, during, and after PTZ-induction. Chi Square test; *p<0.05, ***p<0.001.

- **Opportunities for training and professional development**

Nothing to Report

- **Results disseminated**

Nothing to Report

- **Plan for next reporting period**

Major Task 1: *In vivo* characterization of heterozygous and homozygous NEX-*Tsc2* mice.

To conclude our analysis of excitatory neurons we will perform immunofluorescence analysis of neuronal markers in the brain sections of NEX-Tsc2 mice (already collected). To conclude our analysis of inhibitory neurons will perform RT-PCR and immunofluorescence analysis of markers of specific inhibitory neurons subpopulations, such as parvalbumin or somatostatin. Similarly, we will extend our analysis to include markers of astrocytes, microglia and oligodendrocytes, and we will conduct further Western blot and immunofluorescence analysis to examine potential alterations in signaling transduction, proliferation and cell death.

Major Task 2: *In vitro* analysis of neuronal abnormalities and neuro-glia interactions in NEX-*Tsc2* cultures

We will prepare cortical neuron cultures and conduct the proposed studies focusing on confirming and expanding on the most promising results obtained from the in vivo studies above. We then plan to submit a manuscript for publication toward the end of the next period of support.

4. Impact

- **Impact on the development of the principal discipline of the project**

Our preliminary findings suggest that excitatory and inhibitory synapses may be disrupted as a consequence of Tsc2 deficiency in NEX-Tsc2 KO mice. If confirmed, the non cell-autonomous effect on inhibitory synapses would be a novel and exciting result, pointing to a functional abnormality in synaptogenesis that may cause disinhibition of excitatory neurons in TSC patients as well as animal models. The deficit in the formation of inhibitory synapses could underlie the excessive neuronal activation and the seizures that are frequently associated with TSC.

- **Impact on other disciplines**

Nothing to Report

- **Impact on technology transfer**

Nothing to Report

- **impact on society**

Nothing to Report

5. Changes/problems

Nothing to Report

6. Products

Nothing to Report

7. Participants & Other Collaborating Organizations

- **Individuals who worked on the project:**

Name	*Gabriella D'Arcangelo*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*darcangelo*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. D'Arcangelo planned and supervised this study*
Funding Support:	*New Jersey State teaching line (Rutgers); this DOD award*

Name	*Beth Crowell*
Project Role:	*Senior lab technician*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*12*
Contribution to Project:	*Ms. Crowell managed the NEX-Tsc2 mouse colonies and performed the experiments in the D'Arcangelo lab*
Funding Support:	*this DOD award*
Name	*Anne Anderson*
Project Role:	*Collaborator*
Researcher Identifier (e.g. ORCID ID):	*ANNEEA*
Nearest person month worked:	*.1 calendar months*
Contribution to Project:	*Dr. Anderson supervised the in vivo epilepsy work*
Funding Support:	*DOD, NIH, foundation*
Name	*Amber Levine*
Project Role:	*Research Asst (GS)*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*10*
Contribution to Project:	*Contributed to in vivo epilepsy work*
Funding Support:	*DOD and Foundation funding*
Name	*Julianah Ajose*
Project Role:	*Research Tech II*
Researcher Identifier (e.g. ORCID ID):	*N/A*

Nearest person month worked:	*3*
Contribution to Project:	*Contributed to in vivo epilepsy work*
Funding Support:	*DOD and foundation*

- **Changes in active support**

Nothing to Report

- **Partners organizations**

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Since this is a collaborative award, we have included in this report the results of the work conducted by the Collaborating/Partnering PI (Dr. Anne Anderson, Baylor College of Medicine).

9. APPENDICES: None